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THE MECHANISM OF SOLUTE TRANSPORT BY THE GALL-BLADDER

BY JARED M. DIAMOND
From the Physiological Laboratory, University of Cambridge

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In the preceding paper (Diamond, 1962*a*) it was shown that an absorption of isotonic NaCl, similar to that observed in many other epithelia, is the biological driving force by which the gall-bladder concentrates bile. The present paper will be concerned with the active and passive mechanisms by which solutes cross the gall-bladder *in vitro*; the mechanism of water transport will be deferred to the following paper.

When an epithelial membrane which transports salt and water separates two identical solutions, it has invariably been found that an electrical potential difference (p.d.) is set up across the membrane under at least some experimental conditions. For example, the inside of frog skin becomes positive by up to 130 mV, and this p.d. is due exclusively to active transport of Na^+ inwards. The p.d. carries Cl^- inwards passively, hence active transport of Na is sufficient to account for the transport of NaCl effected by frog skin (Ussing & Zerahn, 1951). In numerous other tissues the side of the preparation towards which NaCl is transported becomes positive by 20–130 mV, owing to active Na transport (e.g. large intestine, foetal stomach, urinary bladder, placenta, rumen, and kidney proximal tubule). In a few cases where the p.d. has the opposite sign, active transport of Cl has been demonstrated (e.g. stomach, bethanechol-stimulated intestine, and adrenaline-stimulated frog skin). Thus, the sign of the p.d. between identical solutions provides a simple test as to the mechanism of salt transport.

In the gall-bladder it has turned out unexpectedly that active salt transport is not associated with any measureable electrical p.d. and that this organ provides the first instance of an apparently neutral NaCl pump. In addition, symmetrical permeability characteristics make the gall-bladder a favourable system for testing the ability of the constant-field equation to predict tracer fluxes.

METHODS

Analytical methods, composition of experimental solutions, and techniques for cannulating gall-bladders from fresh-water fish (roach) and measuring absorption of luminal fluid by weighing have been described in the preceding paper (Diamond, 1962*a*).

Electrical measurements. Salt bridges were constructed of polythene capillaries filled with 3% agar in 0·15M-NaCl or saturated KCl. 'Fine' capillaries were of 50–100 μ internal diameter, 'wide' capillaries of 1·2 mm. The leak resistance through a capillary's polythene wall was found to be greater than 6000 MΩ. DC potentials were measured with a voltage calibrator, pre-amplifying unit, cathode followers, and Cossor oscilloscope; currents by a reflecting galvanometer of maximum full-scale sensitivity $0{\cdot}8 \times 10^{-6}$ A, with appropriate shunts to decrease sensitivity when necessary.

The arrangement for measuring the potential difference (p.d.) across the gall-bladder is depicted in Fig. 1. As the fine bridge had a capillary tip 4·6 cm long and the length of the cannula was 4·2 cm, the tip of the fine bridge was known to be projecting 0·4 cm into the gall-bladder when the entire capillary length of the bridge was within the cannula, and this was occasionally checked visually. The p.d. between the tips of the fine bridge and wide bridge was taken as the setting of the voltage calibrator required to return the oscilloscope

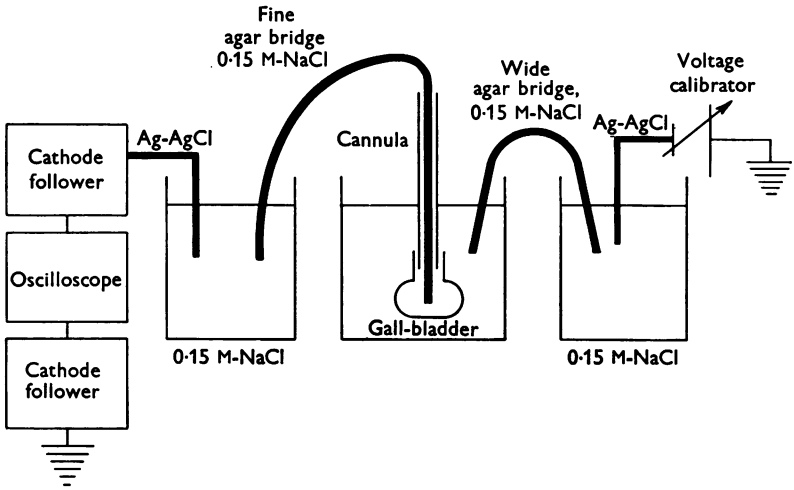

Fig. 1. Arrangement for measuring the electrical potential difference across the gall-bladder. Chlorided silver electrodes were connected to the solutions bathing either side of the gall-bladder by polythene bridges filled with 3% agar in 0·15M-NaCl. The ligatures securing the cannula in the cystic duct are not shown.

trace to earth level. The asymmetry potential of the circuit, taken as the p.d. with the tips of both bridges in the solution outside the gall-bladder, was subtracted from all readings and was generally 0–2 mV. Whenever the solutions inside and outside the gall-bladder differed in chemical composition, the junction potential at the bridges, as determined with a saturated KCl bridge, was also subtracted. Occasionally, saturated KCl bridges were used directly on the preparation to confirm the validity of this procedure, but their regular use was avoided, in order to reduce damage from leakage of KCl.

The same arrangement was used to pass current through the preparation, except that the AgCl electrodes were now connected to a power supply in series with the galvanometer. To measure the resistance of the gall-bladder, both the voltage and current bridges and circuits were set up simultaneously—i.e. four bridges were used, two to pass current and two to record voltage. In this case the tip of the current-passing fine bridge was left half-way up the cannula. The tip of the cannula was approximately central in the gall-bladder, and the tips of the voltage bridges were within a few millimetres of the gall-bladder on either side. Hence the surface of the tissue was approximately equipotential, and the voltage bridges measured virtually the full potential drop across the gall-bladder.

When rapid changes of the outer solution were required, the beaker of solution was lifted away from underneath, and a few seconds later replaced by a fresh beaker. The time when the gall-bladder entered the fresh beaker was taken as the time of changing the solution.

Isotope techniques. Radioactive efflux experiments were carried out by filling the gall-bladder with a radioactive solution, then transferring the preparation every 10 min to a fresh beaker containing 20 ml. (in a few cases 10 ml.) of non-radioactive Ringer's solution. To avoid loss of radioactivity from the system, the gall-bladder was drained only once against the inside of the beaker before weighing at the end of each 10 min period, with a consequent slight reduction in replicability of weighings. At the end of an experiment the luminal solution was recovered by cutting the gall-bladder, as described previously (Diamond, 1962*a*) and the gall-bladder wall was dipped for a few seconds in Ringer's solution to rinse remaining drops of radioactive solution out of the lumen. The sum of the radioactivity in the efflux beakers, recovered luminal solution, rinse solution, and gall-bladder wall usually agreed with the amount introduced to within 5%, proving that no significant amounts had been lost from the system. The total radioactivity in the preparation at the middle of any 10 min period was obtained by adding half the counts/min lost in that period to the counts/min lost in all successive periods and in the recovered luminal solution, rinse solution, and wall. From the total radioactivity at any time, the luminal concentration of the ion under study, and the luminal volume, the luminal specific activity was computed. The absolute efflux (in μmole/hr) at any time is then the quotient of the radioactive efflux divided by the specific activity.

This method of calculation assumes that the back-flux of labelled ions is negligible. This proved to be justified in practice, since the external specific activity was only a fraction of 1% of the luminal specific activity at the end of each 10 min period.

Radioactive influxes were measured directly in a few cases by placing the gall-bladder in a radioactive solution and then recovering the luminal solution, either by the usual method or else by withdrawing most of the luminal contents into a clean polythene capillary and correcting for residual luminal fluid. In influx experiments the luminal specific activity reached, at most, 12% of the external specific activity, and since neglecting the back-flux would thus have involved a small error, the true influx was calculated from the familiar equations for a two-compartment system.

Radioactive samples were generally counted to 10,000 counts or for 10 min: ^{24}Na and ^{42}K by liquid Geiger counting (Keynes, 1958), and ^{82}Br by scintillation counting with the Labgear unit D4105. All samples were corrected for background; ^{24}Na and ^{42}K for counter dead time; and ^{24}Na, ^{42}K, and ^{82}Br for decay, with half-lives of 15·0, 12·4 and 35·9 hr, respectively. These half-lives were confirmed by counting a standard solution on each day of the experiment. Counting of ^{42}K was limited to 2 days to avoid errors from long-lived impurities.

RESULTS

Electrical measurements

Potential differences between two identical solutions

As indicated in the introduction, most salt-transporting epithelia set up potential differences (p.d.s) of 20–130 mV between two identical solutions. When a gall-bladder is bathed on both sides by NaCl Ringer's solution, a transport of NaCl from the lumen to the outside is observed (Diamond, 1962*a*). The p.d. measured under these conditions for twenty-five roach gall-bladders was $-0{\cdot}8 \pm 0{\cdot}6$ mV (i.e. lumen negative; all p.d. measurements will be reported as the potential of the lumen with respect to the outside, and all errors are standard deviations); $-2{\cdot}5$ and $-2{\cdot}3$ mV were

each recorded once, $-1{\cdot}5$ to $-1{\cdot}0$ three times, and all other measurements were between 0 and $-1{\cdot}0$ mV. The corresponding figures for two other species of fish were: bream, $-1{\cdot}0 \pm 0{\cdot}5$ mV (3), pike $-0{\cdot}7 \pm 0{\cdot}3$ mV (2). The p.d. for canine gall-bladder is also usually under 1 mV (E. Grim, unpublished). Since Cu^{2+} gives increased p.d.s in frog skin (Koefoed-Johnsen & Ussing, 1958), $CuSO_4$ was added at 10^{-4}M to both sides of one gall-bladder, but the p.d. remained unchanged at $-1{\cdot}0$ mV. $-0{\cdot}8 \pm 0{\cdot}6$ mV is insignificant in comparison with the usual p.d.s obtained in other salt-pumping preparations, and the chief problem in the mechanism of salt absorption by the gall-bladder will be to explain the lack of a p.d.

There are three ways in which the assumption that the usual transport mechanisms producing p.d.s are present might be reconciled with this failure to measure a p.d.:

(1) Conceivably there might be a masking p.d. of unspecified origin in series with the active-transport p.d. and of equal magnitude and opposite sign, so that the net p.d. across the preparation would then be zero. This masking p.d. should then appear when active transport has been abolished. However, after addition of cyanide and iodoacetate, which have been shown to inhibit the absorptive process completely, the p.d. was $-0{\cdot}4 \pm 0{\cdot}1$ mV (2), i.e. still insignificant. It might be maintained that the hypothetical masking p.d. is also destroyed by cyanide-iodoacetate. Hence in one experiment a partial inhibitor of absorption was tested. A gall-bladder in normal Ringer's solution transported fluid at $11{\cdot}2\,\mu$l./cm^2. hr and gave a p.d. of $-1{\cdot}0$ mV. 3 mV cyanide in the outer solution inhibited absorption by 61 %, while the p.d. was now $-0{\cdot}3$ mV, i.e. still insignificant. Since it is more likely that the hypothetical masking p.d. does not exist than that it too has been inhibited by 61 %, one may conclude that changes in pumping rate are not associated with changes in the p.d.

(2) If both an anion and a cation were transported actively, independently, in the same direction, and at fortuitously comparable rates, the two transport p.d.s would be of opposite sign and would tend to cancel each other. This situation arises in three other preparations that may give low p.d.s: small intestine and *in vivo* frog skin transport both Na and Cl, and stomach transports H^+ and Cl. However, in all three cases it has been possible to observe a p.d. of the sign and magnitude expected for one pump by eliminating the other. For instance, in frog stomach the anion sulphate is not pumped, so that the anion pump is inoperative in sulphate solutions, and a p.d. of the sign expected for an independent H^+ pump is observed (Heinz & Durbin, 1959). On the other hand, thiocyanate eliminates H^+ secretion, and in (chloride) Ringer's solution increased potentials of the sign expected for an independent Cl pump appear (Durbin & Heinz, 1958). Similarly, the Na and Cl pumps of frog skin can be shown to function

independently *in vivo* (Barker Jørgensen, Levi & Zerahn, 1954), and *in vitro* the independent sodium pump can produce p.d.s of up to 152 mV in sulphate solutions (Koefoed-Johnsen & Ussing, 1958).

Thus, one might suppose that the gall-bladder happens to transport Na and Cl independently at the same rates. Since the anions SO_4 and CH_3SO_4 are transported with only low efficiency (Diamond, 1962*a*), an independent Na pump would produce a lumen-negative p.d. in Na_2SO_4 and $NaCH_3SO_4$ solutions. The actual p.d. in Na_2SO_4 was $-1{\cdot}7 \pm 0{\cdot}9$ mV (12) and showed no increase after several hours in sulphate. These p.d.s remained virtually unchanged after cyanide, which inhibits by 67 % the small residual rate of absorption observed with Na_2SO_4. In $NaCH_3SO_4$ solution the p.d. was $-1{\cdot}5$ mV. Similarly, an independent chloride pump would create a lumen-positive p.d. in TEACl solution, since TEA is absorbed only with low efficiency; but the measured p.d. was $-0{\cdot}3 \pm 0{\cdot}3$ mV (2).

(3) The observation that the gall-bladder yields no p.d. is not quantitative, in that one does not know how many millivolts independent Na and Cl pumps would produce, if they existed, in the gall-bladder. For example, if the passive permeabilities to Na and Cl were sufficiently high, the Na and Cl pumps might generate a voltage too low to detect with confidence, but still might transport NaCl at the observed rates. To compute pump p.d.s, one must know either the relative ionic permeabilities, as measured from diffusion potentials, and the electrical resistance; or else the passive fluxes of ions, as measured by radioactive tracers. Accordingly, the measurement of these quantities is described in the following sections.

Diffusion potentials

Salt–sucrose substitutions. When both sides of the gall-bladder were initially bathed in chloride (sixteen experiments), sulphate (eight experiments), bromide (five experiments), or methyl sulphate solutions (one experiment), and when the sodium salt on one side of the gall-bladder was partially replaced by sucrose, that side went positive with respect to the other. Figure 2 depicts the results of an experiment in which a gall-bladder was subjected to six such changes of solution in NaCl Ringer's solutions (the curve drawn through the experimental points is theoretical and will be discussed later).

When a gall-bladder had been poisoned 45 min with cyanide–iodoacetate, salt–sucrose substitutions gave the same p.d.s as those obtained before poisoning, although the absorptive capacity as tested by the weighing method was completely inhibited by the poison. After 2 hr in cyanide-iodoacetate the p.d.s were 40 % below the original values, presumably because of general deterioration of the preparation. However, since cyanide-iodoacetate has no primary effect on these p.d.s, they must be

unrelated to pumping. When the substitutions were effected in the lumen, the p.d.s remained stable for as long as they were observed (up to 50 min). In substitutions of short duration the p.d. changed in sign but remained about the same in magnitude when the solutions were reversed. However, if more than 50 % of the sodium salt in the outer solution was replaced, the p.d. began to decay after 10–20 min, eventually declining to the values found for junction potentials in free solution. At this point the gall-bladder

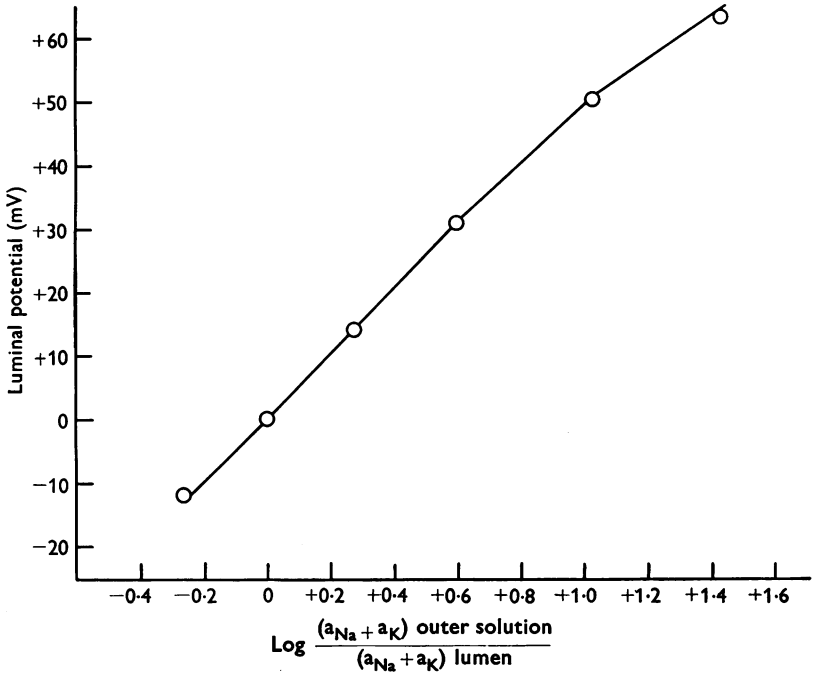

Fig. 2. Diffusion potentials arising from a NaCl concentration gradient across the gall-bladder. Abscissa, the activity ratio for the sum of Na and K (a positive value of the logarithm means that [Na] and [Cl] are higher in the outer solution than in the lumen). Ordinate, potential of lumen with respect to outer solution. ○, experimental points. The curve is the potential difference calculated from the constant-field equation, assuming $P_K : P_{Na} : P_{Cl} = 1{\cdot}00 : 1{\cdot}00 : 0{\cdot}04$.

NaCl in one bathing solution was partially replaced by sucrose by mixing solutions *A* and *D* (Table 2, Diamond, 1962*a*); the other bathing solution was NaCl Ringer's solution (*A*). All solutions contained 4·5 mM-K, and [Cl] was always 5·6 mM greater than [Na].

was neither semi-permeable nor capable of absorption—i.e. it was physiologically dead. The decline in p.d. must have resulted from the experimental manipulation involved in changing the luminal solution and from the cumulative damaging effects of low salt concentrations in the outer solution, since gall-bladders kept for 4 hr in solutions from which no salt was removed could give normally high diffusion potentials at the end of this time.

Accordingly, quantitative consideration in the discussion of the normal gall-bladder will be restricted to the following values, all measured before the p.d. for 50 % replacements of sodium salts had declined below 11·5 mV: 50 % of NaCl on one side replaced by sucrose, 14 ± 1 mV (7); 80 %, 31 mV; 95 %, 50 mV (replacement only in lumen; this substitution in the outer solution gave p.d.s which declined continuously towards the junction potential); 100 %, 63 mV (only in lumen); in NaBr solution, 13 mV for 50 % replacement, 58 mV for 100 % (only in lumen); in Na_2SO_4 solution, 15 ± 1 mV (5) for 50 % replacement; in $NaCH_3SO_4$ solution, 14 mV for 50 % replacement. In the only other species tested, pike, 50 % replacement of NaCl in the outer solution left the lumen 10 mV negative.

K–Na substitutions. When half the Na on one side of the gall-bladder was replaced by K (keeping other ions constant), the p.d. was always 0 ± 2 mV. Thus, in one experiment where the anion was sulphate, the p.d. was − 0·2 mV with normal sulphate solution (4·5 mM-K) on both sides, and replacing half the outside Na with K gave + 2 mV. Restoring normal sulphate solution to the outside and replacing half the Na on the inside with K gave − 1·7 mV. 50 % replacement of luminal Na made the lumen negative in two gall-bladders, positive in two others, when the anion was sulphate (average + 0·2 mV): 1 mV negative in one experiment with bromide; 1·5 mV positive in one experiment and zero in the other with chloride. In each experiment the sign of the small p.d., if there was any, changed when the solutions were reversed, as in the sulphate experiment just described. Replacement of all luminal Na by K gave + 0·3 mV in NaCl Ringer's solution. These p.d.s remained stable for as long as they were observed (up to 1 hr), and no changes were noticed after cyanide-iodoacetate.

Thus, with all three anions Na–K substitutions produce either no change or variable insignificant changes in the p.d. Then either P_K and P_{Na} (P is the relative permeability coefficient) are approximately the same, or else both are much smaller than the anion permeabilities.

Anion substitutions. With NaCl Ringer's solution as the outer solution and sulphate in the lumen, the p.d. was − 0·7 mV, and after 3 mM cyanide, − 0·3 mV. In the reverse case (sulphate as the outer solution and chloride in the lumen), + 0·5 mV and + 1·5 mV were observed with two gall-bladders.

Tetraethyl ammonium chloride (TEACl). If the gall-bladder was initially in TEACl solution and half the TEACl was then replaced by sucrose on one side, that side went negative by 4 mV. With all the TEACl in the lumen replaced by sucrose the p.d. was − 10 mV. The same gall-bladders gave the usual p.d.s in NaCl solutions with sucrose substitutions.

Chloroform reduced all these diffusion potentials within a few minutes to the values found for liquid junction potentials. The isolated serosa,

prepared by stripping the mucosa off the inside of the gall-bladder, also gave only junction potentials. The mucosa proved too friable to survive in isolation and disintegrated as it was separated from the serosa.

Relative permeability coefficients. In most biological preparations which transport salt a p.d. associated with transport is set up even between two identical solutions. Changes in p.d. as a result of changes in one bathing solution may thus represent not only diffusion potentials (in the ordinary sense of steady-state p.d.s due to ionic concentration differences across an inert membrane) but also changes in the transport p.d. Analysis of diffusion potentials in the gall-bladder is considerably simplified by the fact that this preparation shows no transport p.d. The p.d.s observed between two dissimilar solutions are unaffected by cyanide-iodoacetate, which completely inhibits salt transport, and they may thus be regarded as pure diffusion potentials.

Three obvious qualitative conclusions may be drawn from the diffusion potentials in fresh gall-bladders: (i) The permeability to all anions is less than the permeability to Na (P_{Na}), since the more dilute solution is always electrically positive when the gall-bladder separates two solutions of the same sodium salt at different concentrations. (ii) Since K–Na substitutions have virtually no effect on the p.d. and since P_{Na} is larger than the anion permeabilities, P_K must equal P_{Na} within experimental error. (iii) Strictly speaking, the gall-bladder, like other epithelial 'membranes', consists of two layers of cell membranes in series—the inner and outer membranes of the epithelial cells, facing the lumen and serosa, respectively. However, the gall-bladder must be symmetrical: i.e. the relative permeability coefficients must be the same in these two membranes, since the p.d. from a change of solutions in the lumen has the opposite sign and same magnitude as the p.d. from a change of the outer solution. This simple situation may be exceptional for biological epithelia. For instance, the inner membrane of frog skin behaves like a potassium electrode, and the outer as a sodium electrode (Koefoed-Johnsen & Ussing, 1958); and a similar asymmetry is characteristic of kidney proximal tubule (Giebisch, 1961).

In a membrane permeable to more than one ionic species, quantitative interpretation of diffusion potentials in terms of relative permeability coefficients is possible only after specific assumptions about the membrane structure have been made. For many purposes the so-called constant-field equation (after Goldman, 1943; Hodgkin & Katz, 1949) has proved adequate:

$$E = 2{\cdot}3\frac{RT}{F}\log\frac{P_K\gamma^o_K[K]^o + P_{Na}\gamma^o_{Na}[Na]^o + P_{Cl}\gamma^i_{Cl}[Cl]^i}{P_K\gamma^i_K[K]^i + P_{Na}\gamma^i_{Na}[Na]^i + P_{Cl}\gamma^o_{Cl}[Cl]^o}, \quad (1)$$

where γ is a single-ion activity coefficient;

square brackets refer to concentrations;

superscripts l and o refer to the luminal and outer solutions respectively; and

E is the potential of the lumen with respect to the outer solution.

The factor 2·3 RT/F is 58 mV at 25° C. It will be assumed that the small quantities of Ca, Mg, and phosphate present on both sides of the membrane at the same concentration have negligible effects on the diffusion potential. γ for a single ion (abbreviated γ_+ or γ_-) may be computed from published values for $\gamma_\pm$ of salts (in Harned & Owen, 1950; Robinson & Stokes, 1959) by the definition

$$\frac{\ln \gamma_+}{z_+^2} = \frac{\ln \gamma_-}{z_-^2} = \frac{\ln \gamma_\pm}{z_+ z_-}.$$

For a 1:1 salt, $\gamma_+ = \gamma_- = \gamma_\pm$. For Na_2SO_4, a 2:1 salt, $\gamma_+^2 = \sqrt{\gamma_-} = \gamma_\pm$.

As a sample calculation one may consider NaCl Ringer's solution in the outer solution and 50 % NaCl + 50 % sucrose in the lumen, which yields 14 mV. The relevant concentrations are: $[Na]^o = 144$ mM, $[K]^o = 4{\cdot}5$, $[Cl]^o = 149{\cdot}6$, $[Na]^l = 72$, $[K]^l = 4{\cdot}5$, $[Cl]^l = 77{\cdot}6$. Since $\gamma_{KCl} = \gamma_{NaCl}$ and [K] is in any case small, the outer solution approximates to 149 mM-NaCl, in which $\gamma_\pm = 0{\cdot}758 = \gamma_{Na}^o = \gamma_K^o = \gamma_{Cl}^o$. The lumen approximates to 77 mM-NaCl, in which $\gamma_\pm = 0{\cdot}795 = \gamma_{Na}^l = \gamma_K^l = \gamma_{Cl}^l$. Since one is concerned only with relative permeabilities, P_{Na} is taken as 1·00, by definition. Then P_K is also 1·00. Substituting these values in the constant-field equation yields $P_{Cl} = 0{\cdot}05$. When 50 % replacement is in the outer solution, the result is the same, since the p.d. is of the same magnitude and opposite sign.

One obtains similarly: $P_{Cl} = 0{\cdot}04$ for 80, 95 and 100 % unilateral replacement of NaCl (by sucrose); $P_{Br} = 0{\cdot}08$ for 50 % replacement of NaBr, 0·06 for 100 %; $P_{CH_3SO_4} = 0{\cdot}05$ for 50 % replacement of $NaCH_3SO_4$; $P_{TEA} = 0{\cdot}02$ for 50 or 100 % replacement of TEACl. Since published activity coefficients are apparently not available for $NaCH_3SO_4$ or TEACl, γ for these two salts has been assumed to be identical with γ for NaCl. If their activity coefficients actually drop more sharply with concentration than does γ_{NaCl}, $P_{CH_3SO_4}$ and P_{TEA} should be lower than the calculated values. For 50 % replacement of Na_2SO_4, P_{SO_4} comes out to be zero. That is, the predicted E with only Na and K terms in the constant-field equation is 14·9 mV, which does not differ from the experimental value of 15 ± 1 mV. A further check on P_{SO_4} is provided by the case of NaCl Ringer's solution in the lumen and sulphate in the outer solution (or vice versa). If P_{SO_4} is assumed to be zero, the constant-field equation as given above (eqn. (1)) need be modified only by omission of the term $P_{Cl}\gamma_{Cl}^o[Cl]^o$ and predicts 0 mV. This agrees well with the average experimental value of $0{\cdot}9 \pm 0{\cdot}5$ mV (3), confirming the assumption that P_{SO_4} cannot be distinguished experimentally from zero.

Thus, the relative permeability coefficients are practically independent of concentration, and have the following average values;

$$P_{Na} = 1{\cdot}00, \quad P_K = 1{\cdot}00, \quad P_{Br} = 0{\cdot}07, \quad P_{CH_3SO_4} = 0{\cdot}05,$$
$$P_{Cl} = 0{\cdot}04, \quad P_{TEA} = 0{\cdot}02, \quad P_{SO_4} = 0{\cdot}00.$$

The theoretical curve of Fig. 2 is drawn on the assumption that $P_{Na} = P_K = 1{\cdot}00$, $P_{Cl} = 0{\cdot}04$ for all NaCl–sucrose substitutions. The good agreement with the experimental points, all derived from one gall-bladder,

simply means that all concentration gradients experimentally tested give this permeability ratio in the constant-field equation. Thus, in K–Na solutions the gall-bladder approximates a reversible electrode for univalent cations. The slight leak to monovalent anions reduces the p.d. for an NaCl activity ratio of 10 from the theoretical value of 58 to 50 mV.

Diffusion potential transients

The p.d.s discussed so far have been steady-state values. When a fresh solution was introduced into the lumen, the p.d. was found to have reached this steady-state value by the time of the first experimental measurement 20 sec later and then remained constant to within 0·5 mV, even when the p.d. change was 60 mV. However, when the outer solution was changed, large diphasic transient effects were observed, lasting up to several minutes and consisting of an immediate p.d. jump followed by a gradual progress to the steady-state value. Figure 3 illustrates these transients when a gall-bladder whose lumen is filled with NaCl solution is transferred from an outer solution of NaCl to Na_4SO_4 and back again. In the steady state there is no change in the p.d. (about 0 mV), but the transient involves a sudden ± 8 mV step and gradual return to 0. K–Na substitutions, which also involve no change in the steady-state p.d., gave qualitatively similar transients.

Figure 4 depicts transients for sucrose–NaBr replacements, which do involve a change in the steady-state p.d. (the significance of the theoretical curve will be discussed later). During these replacements the lumen contained NaBr solution. When the outer solution was changed from NaBr to 20 % NaBr + 80 % sucrose, the p.d. promptly increased from $-0{\cdot}5$ to $+3$ mV, then dropped gradually to $-16{\cdot}5$ mV. When NaBr was restored, the mirror image of this transient took place, but somewhat more rapidly. For NaCl–sucrose replacements the results were qualitatively similar. In transfers from Na_2SO_4 to Na_2SO_4 + sucrose mixtures the immediate jump was a decrease in p.d. followed by a gradual and much larger drop, and restoration of Na_2SO_4 produced an initial sudden rise followed by a gradual larger rise back towards zero.

To facilitate comparison, the 'half-time' of the transients in salt–sucrose substitutions was defined as the time required for the p.d. to rise or fall half way from the value observed immediately after the change of solutions to the steady-state value. It was noticed that the longer half-times were associated with thicker-walled gall-bladders, and most values ranged from 9 to 50 sec. Half-times for p.d.s becoming increasingly negative were greater than for p.d.s returning to zero, and this difference was exaggerated as more salt was replaced by sucrose. For instance, one gall-bladder was taken through the following sequence of changes: NaBr to 50 % NaBr + 50 %

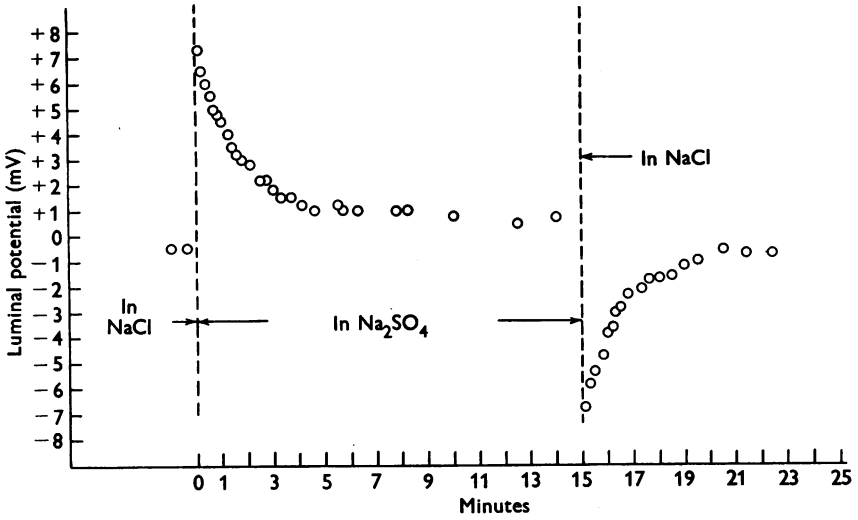

Fig. 3. Diffusion potential transients in NaCl and Na_2SO_4 solutions. The luminal solution remained NaCl Ringer's solution throughout. At $t = 0$ the outer solution was changed from NaCl to Na_2SO_4 solution and at $t = 15$ min, Ringer's solution was restored. [Na] is the same in both solutions.

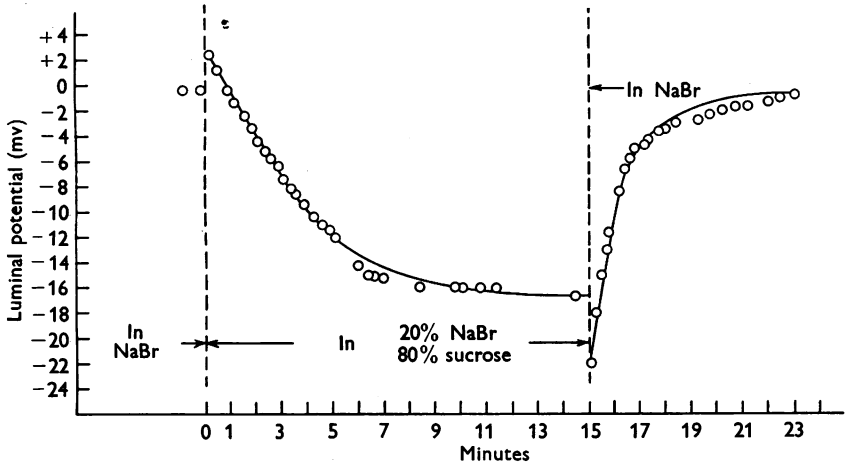

Fig. 4. Diffusion potential transients for NaBr concentration changes in the outer solution. The luminal solution remained NaBr throughout. At $t = 0$, 80% of the NaBr in the outer solution was replaced by sucrose, and at $t = 15$ min, 'NaBr Ringer's solution' was restored. ○, experimental points. The curves are theoretical, calculated on the assumption that the transients are due to serosal diffusion delays (see text).

sucrose; back to NaBr; then into 20 % NaBr + 80 % sucrose; finally back to NaBr. The respective half-times were in the ratio: 1·00:0·67:1·53: 0·49. Half-times were longer in sulphate than in bromide or chloride Ringer's solutions.

It is highly improbable that these transients could involve changes in the actual ionic permeability coefficients, since the coefficients were the same in all steady states observed. Presumably the semi-permeable membranes responsible for the steady-state p.d.s are the inner and outer membranes of the epithelial cells. Since the cells are in direct contact with the luminal solution, but separated from the outer solution by the connective tissue of the serosa, it might be suspected that the transients arise from diffusion delays in the serosa, as no transient effects were encountered for luminal changes. As soon as the outer solution is changed, a junction potential would be set up between the new outer solution and the extracellular fluid of the serosa, which would be virtually identical with the old outer solution. In fact, the p.d. change in the sudden jump at zero time is always approximately equal to the junction potential between the new and old outer solutions. The NaCl–Na_2SO_4 transients, which involve no change in cation concentration and hence also in the steady-state p.d., consist solely of the prompt establishment of this 8 mV junction potential and its gradual decay as the new outer solution diffuses into the serosa. In salt–sucrose substitutions this gradual diffusion not only wipes out the junction potential but also changes the cation concentration at the outer membrane of the epithelial cells (facing the serosa) and thereby establishes the new steady-state p.d.

For mathematical treatment of this diffusion problem the gall-bladder may be represented by an infinite plane sheet, since its thickness is negligible compared to its radius. The appropriate equation is:

$$\frac{\partial C(x,t)}{\partial t} = D\frac{\partial^2 C(x,t)}{\partial x^2},$$

and the boundary conditions: $C = C_1$, $x > l$; $t = 0$, $C = C_0$, $l > x > 0$; $D = 0$, $x = 0$.

C is the concentration of sodium salt in the serosal extracellular space;

C_1 and C_0 the concentration in the new and old outer solutions, respectively;

D the diffusion coefficient of the salt in the serosa (not necessarily the same as the value in free solution);

x is the depth in the serosa measured from the epithelial cells, i.e. $x = 0$ is the border between the serosa and epithelial cells, and $x = l$ is the border between serosa and outer solution; and

l is the thickness of the serosa.

The solution to this problem (see Carslaw & Jaeger, 1947, for a similar problem in heat conduction) is:

$$C(x,t) = C_1 + \frac{4}{\pi}\sum_{n=0}^{\infty}[\exp\{-D(2n+1)^2\pi^2 t/4l^2\}]\frac{(C_1 - C_o)(-1)^{n+1}}{2n+1}. \quad (2)$$

The theoretical curves for the transients of Fig. 4 have been calculated from the constant-field equation and from $C(0, t)$ in eqn. (2), with D/l^2 obtained from the half-time of the

transient. In general, the theoretical curves fit the experimental points well. It may be calculated that the half-times ought to be in the ratio of 1·22:1·00:0·69:0·55 for the following changes of outer solution, respectively: 100 % salt to 20 % salt (+80 % sucrose), 100 % to 50 %, 50 % to 100 %, and 20 % to 100 %. The experimental ratios obtained for two gall-bladders in NaBr + sucrose solutions are in the same order and rather similar quantitatively: 1·58:1·00:0·63:0·54 and 1·53:1·00:0·67:0·49. On one gall-bladder transients were measured in both NaCl and Na_2SO_4 solutions, and yielded half-times in the ratio of 1·00:1·35 (Na_2SO_4 slower). Since the half-time is inversely proportional to the diffusion coefficient D, $D_{Na_2SO_4}/D_{NaCl}$ in the serosa is $1/1{\cdot}35 = 0{\cdot}74$, as compared to 0·77 in free solution. Hence the serosa is not semi-permeable but merely retards the diffusion of both salts proportionately. If the effect of the unknown thickness of unstirred layer of solution just outside the serosa is ignored, and if l is taken as just the thickness of the serosa itself, one obtains minimum values around 10^{-6} cm²/sec for D in most gall-bladders. D thus decreases by at most an order of magnitude in the serosa as compared to free solution, presumably due to a reduction in mean free path. Similar reductions of D have been observed in other extracellular spaces, such as inter-fibre water of frog sartorius muscle (Keynes, 1954), rabbit tendon (Cotlove, 1954), and rat diaphragm (Krnjević & Mitchell, 1960).

Thus, most properties of the transients—their diphasic form, the dependence of the half-times on the sequence of solutions, the ratio of their half-times for NaCl and Na_2SO_4 solutions, their time course, and the absence of transients for luminal substitutions—are in accord with the notion that the serosa presents an indiscriminate retarding force to diffusion. The diffusion coefficient of all ions is reduced by a factor of somewhat less than 10, as compared to free solution. The transients are also useful in confirming the conclusion, drawn from steady-state p.d.s, that the permeability of the gall-bladder is symmetrical. During K–Na and salt–sucrose substitutions in the bathing solutions the composition of the intracellular fluid will also be changing. However, if and only if two membranes in series have identical relative permeability coefficients will a diffusion potential arising from concentration differences between the two outermost compartments be independent of the composition of the middle compartments (corresponding to the intracellular fluid). Thus, the facts that there are no transients for luminal substitutions, and that serosal transients are qualitatively and quantitatively attributable to diffusion delays, means that the inner and outer membranes of the epithelial cells must have the same relative permeability coefficients.

Electrical resistance

When a constant direct current was passed through the gall-bladder, the p.d. assumed a constant new value by the time of the first experimental observation—i.e. within a few seconds. No transient effects were noted, and the p.d. continued to remain constant if the current was continued for several minutes. The change in p.d. was directly proportional to the current up to the highest currents passed (producing p.d.s of 25 mV), and the proportionality constant was the same when the direction of the current

was reversed. The gall-bladder thus behaves as a simple ohmic resistance, with an average value of $113 \pm 16\ \Omega\,cm^2$ (3). The resistance was unchanged when chloride was replaced by sulphate in either the luminal or outer solution.

Radioactive measurements

^{24}Na

Figure 5 depicts the efflux of Na from a gall-bladder as measured by ^{24}Na. In the course of 3 hr the efflux tends to decline slowly with time. However, upon addition of cyanide-iodoacetate the efflux drops more rapidly, and fluid absorption as measured by loss of weight ceased apparently simultaneously. From the weighing experiments already described

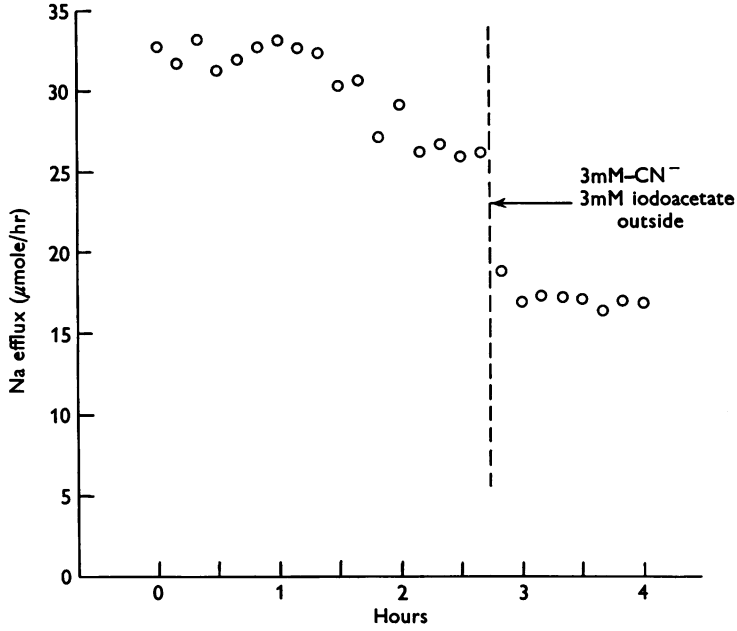

Fig. 5. Effect of cyanide-iodoacetate on Na efflux in the gall-bladder. Ordinate, Na efflux as calculated by means of ^{24}Na. At t = 165 min 0·58 ml. of a neutral isotonic solution of 103 mM-NaCN + 103 mM iodoacetic acid was added to 20 ml. of the outer solution.

(Diamond, 1962*a*), one would expect the loss of water to be accompanied by NaCl in isotonic proportions—i.e. the observed weight loss of 37·9 mg/hr implies a net Na flux of 5·9 μmole/hr (1·6 μmole/cm².hr), in good agreement with the observed decline in the efflux of 7·2 μmole/hr (1·9 μmole/cm².hr). The residual efflux of 17·0 μmole/hr must be equal to the influx and represent the passive Na permeability. The effect of cyanide-iodoacetate was tested in one other similar experiment in normal NaCl Ringer's

solution, and the expected and observed drops in Na efflux were 2·0 and 2·4 μmole/cm^2.hr, respectively. The simplest explanation of the effect of poisoning is thus that it abolishes the active net efflux of Na without affecting the passive permeability.

The Na influx was calculated indirectly by subtracting the net flux of Na (from the weight loss, assuming the absorbate to be isotonic NaCl) from the total measured efflux. Since this calculated influx gradually declined in some experiments by up to 35 % during the course of several hours, the values of the influx at the beginning and end of the experiment were averaged. For eleven gall-bladders the average influx was 8·4 $\pm$ 2·4 μmole/cm^2.hr (11). In one gall-bladder where ^{24}Na was added to the outer solution and samples were collected from the lumen, the influx was measured directly as 6·6 $\pm$ 0·5 μmole/cm^2.hr (average of four experimental periods), which falls within the range of indirectly measured values. In the same experiment replacement of all luminal Na by K produced no alteration in the influx, and cyanide-iodoacetate had little effect on the influx while reducing the rate of weight loss to zero. The indirectly measured influx was 6·4 μmole/cm^2.hr in sulphate solution and 7·7 μmole/cm^2.hr when all K was removed from the lumen. Both values fall within the normal range. However, the influx increased by a factor of 4·7 after a gall-bladder had been chloroformed.

Since the net flux of water in normal Ringer's solution is 15·3 μl./cm^2.hr and the absorbate is virtually isotonic NaCl, the net efflux of Na is 2·4 μmole/cm^2.hr. Thus, for every sodium ion transported actively outwards, 8·4/2·4 = 3·5 sodium ions cross the gall-bladder passively in either direction.

^{82}Br

Br efflux was similarly measured with ^{82}Br on four gall-bladders in bromide solution, and remained relatively constant over the course of 2 hr. Subtraction of the net flux (calculated from weight loss on the assumption of absorbate isotonicity) from the efflux gave an average value of 12·2 $\pm$ 2·5 μmole/cm^2.hr (4) for the influx. When the mucosa was stripped off the inside of a gall-bladder, the efflux through the resulting serosa was 206 μmole/cm^2.hr—i.e. sixteen times higher. From the known thickness of this serosa, it would have a D_{Br} of 6×10^{-6} cm^2/sec (27 % of the free solution value) if it were uniform.

The flux measurement in the serosal preparation also shows that omission of stirring in the luminal solution does not introduce any significant error into flux measurements on the complete gall-bladder. The method of calculating flux rates from radioactive exchange carries the implicit assumption that the membrane separates perfectly stirred compartments. However, failure to achieve this condition experimentally in the lumen would be serious only if the resistance of the unstirred layers provided a significant fraction of the total

resistance to the flux of the isotope. For the gall-bladder, one can calculate from diffusion equations that this effect should be negligible for K, Na, and Br; and this is confirmed experimentally by the sixteenfold increase in flux that occurs on simply removing the mucosa. Since radioactive fluxes are inversely proportional to effective resistances and since the lumen of the serosal preparation is still unstirred, the mucosa must provide 15/16 of the total resistance to Br in the intact gall-bladder.

At first sight it seems contradictory that the passive Br fluxes are slightly larger than the passive Na fluxes (12·2 compared to 8·4 μmole/cm^2.hr), although P_{Na} is 14 times P_{Br} from diffusion potential measurements. Similar discrepancies, involving tracer fluxes which are larger than those predicted from electrical conductivities, have been observed in other preparations (e.g. chloride in the stomach; Hogben, 1955) and taken to mean that a portion of the tracer flux is electrically non-conducting. The hypothesis of exchange diffusion attributes this phenomenon to the presence in the membrane of a carrier, which can reversibly bind an ion present in the external solution, e.g. Br. If the carrier is supposed capable of crossing the membrane only as a Br-complex, the carrier must convey equal quantities of Br in both directions. Br will then be able to cross the membrane in a non-conducting form, giving rise to higher tracer fluxes but of course no net flux.

An experimental test of this possibility depends upon measuring the ratio of the one-way tracer fluxes. Ussing (1950) has shown that if ions of a particular species cross a membrane without mutual interaction, an equation of the following form should hold:

$$\frac{M_e}{M_i} = \frac{C^l}{C^o} \exp(zF\Delta\psi/RT), \tag{3}$$

where M_e = efflux; M_i = influx; C^l = luminal concentration; C^o = outer concentration; $\Delta\psi$ = p.d. Since active transport of bromide would itself cause the flux ratio to deviate from this prediction, the experiments were carried out on gall-bladders which had been poisoned with cyanide-iodoacetate and were no longer transferring fluid between two identical solutions. The gall-bladder was filled with 50% NaBr solution, 50% sucrose solution (77·8 mM-Br) and placed in normal bromide solution (149·6 mM-Br). Influx experiments were then performed on two gall-bladders, and efflux on two others, with concomitant measurement of the p.d. The average value of the p.d. was 8·1 mV, the influx, 4·8 μmole/cm^2.hr, and the efflux 2·6 μmole/cm^2.hr. Equation (3) then predicts a flux ratio of 2·7, whereas the experimental value is 1·8. This deviation from the predictions based on simple diffusion is in the direction to be expected from the presence of carrier-mediated exchange diffusion, which would add a constant term to both the influx and efflux, thereby reducing the flux ratio. Since it was also found that poisoning promptly inhibits exchange diffusion

and eventually increases ordinary diffusion of Br, larger deviations from eqn. (3) would have been noted but for the side-effects of poison.

^{42}K

In two experiments in NaCl Ringer's solution (4·5 mM-K) the K efflux was measured with ^{42}K as 0·26 and 0·34 μmole/cm^2.hr. Cyanide-iodoacetate reduced fluid absorption to zero in the first experiment, but the K efflux remained unchanged at 0·26 μmole/cm^2.hr. This is what one would expect from the previous observation that K is not actively absorbed. A net increase of luminal K was observed in both ^{42}K experiments. If this gain was due to the influx across the gall-bladder exceeding the efflux, the influx was 0·32 and 0·39 μmole/cm^2.hr, respectively. However, the net increase of K in the lumen represented only 17 and 40 %, respectively, of the total amounts of K in the gall-bladder wall at the end of the experiments. Thus, one might also assume that this extra K leaked into the lumen from cells of the gall-bladder, and that the influx and efflux across the gall-bladder are equal. Hence the average efflux is 0·30 μmole/cm^2.hr, and the average influx somewhere between 0·30 and 0·36 μmole/cm^2.hr.

Radioactive transients

The efflux measurements described so far were made on gall-bladders which had been filled three times in succession with radioactive solution, a procedure requiring up to 5 min. When ^{24}Na was suddenly added to the lumen of a gall-bladder and the outer solution was rapidly changed at intervals of 10 sec or less, the Na efflux rose from zero to its steady-state value with a half-time of 64 ± 5 sec (3) (Fig. 6). Hence a finite time is required for Na to cross the gall-bladder. A similar delay was also observed with ^{82}Br. However, the efflux reached its steady-state value within 10 sec in a chloroformed gall-bladder.

Conceivably this delay might be due to the same cause as the transients in diffusion potentials—i.e. the restricted diffusion of salts in the serosa. However, the estimates of serosal diffusion coefficients already obtained permit one to calculate that the half-time should then be only 1/8 of the experimental values. As a more direct test of the role of serosal diffusion delays, a ^{82}Br efflux transient on the isolated serosa of a gall-bladder whose mucosa had been stripped off gave a half-time of only 7 sec. Hence almost all the delay in a whole gall-bladder must originate in the mucosa (epithelial-cell layer). If the effect there is solely one of retarded diffusion, the diffusion coefficient of Na would have to be nearly 50 times lower than its value in free solution. This assumption must be regarded as improbable until proved otherwise, since Na and K diffusion coefficients inside other cells differ relatively little from their values in free solution (nerve,

Hodgkin & Keynes, 1953; the alga *Nitella*, E. A. C. MacRobbie, unpublished; striated muscle, Harris, 1954). A likelier explanation is that in crossing the thickness of the gall-bladder ^{24}Na (or ^{82}Br) must traverse *two* cell membranes (the membranes at the luminal and serosal faces of the epithelial cells), between which lies a pool of initially unlabelled Na or Br, the intracellular contents. Since ^{24}Na leaving the epithelial cells will have the specific activity of this pool, the flux of ^{24}Na across the gall-bladder will attain a constant value only when the pool specific activity has risen to its steady-state value, determined by the relative values of the Na fluxes across the luminal and serosal faces.

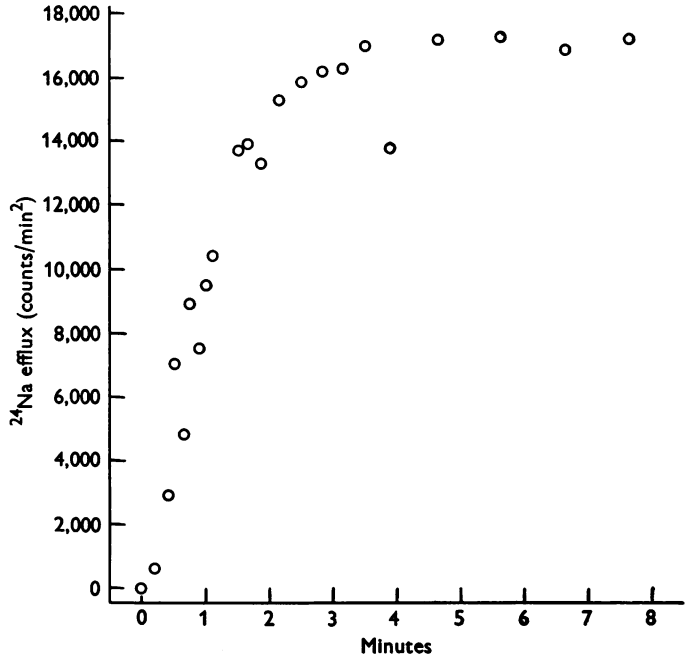

Fig. 6. ^{24}Na efflux transient. At $t = 0$ ^{24}Na was added to the lumen. The outer solution was then changed at short intervals, and the counts/min appearing in the outer solution in each collection period were divided by the duration of the collection period to give the efflux in counts/min^2.

Similar ^{24}Na transients have been observed in frog skin, toad skin, and toad urinary bladder by Hoshiko & Ussing (1960) and have also been interpreted in terms of a pool effect. They show that the pool 'size' (the amount of Na in the pool) is given by MR/k, where the rate constant k is derived from a semi-logarithmic graph of the transient, M is the steady-state efflux, and R is a function of the one-way fluxes at each face of the cell. For a given value of the net flux, R and the pool size are minima if

the passive one-way fluxes of Na across the luminal and serosal faces of the epithelial cells are equal, as appears to be approximately true for the gall-bladder from other evidence.

Minimum pool sizes were calculated for the experimental gall-bladder transients according to this formulation, i.e. on the assumption that the luminal and serosal membranes have equal resistances. The actual amounts of intracellular ions were computed from the values for the intracellular concentration and extracellular space given previously. In each experiment the intracellular amounts and experimental minimum pool sizes were rather similar: 0·41 vs. 0·27, and 0·43 vs. 0·38 μmole/cm^2 for Na and two gall-bladders in NaCl Ringer's solution; and 2·14 vs. 2·28 μmole/cm^2 for Br and a gall-bladder in bromide solution. Thus, the effect of the epithelial cell contents acting as a pool of non-labelled ions is of the right order of magnitude to explain the observed delay in the efflux transients. The fact that the delay is reduced to less than 10 sec in a gall-bladder in Ringer's solution saturated with chloroform, which would be expected to destroy cell membranes and thus the pool effect, is further evidence for this explanation. Ions must therefore pass through and not between the epithelial cells in crossing the normal gall-bladder. The fact that the ^{24}Na efflux transient remained unchanged after cyanide-iodoacetate means that Na to be actively transported is taken from the intracellular pool rather than sped across or between the cells without exchanging.

DISCUSSION

Comparison of constant-field predictions and experimental fluxes

When a membrane separates two identical solutions, one can use membrane-resistance and relative-permeability coefficients to predict tracer fluxes if the ion in question crosses the membrane by simple diffusion without interaction effects: i.e. in the absence of exchange diffusion and single-files. Although the gall-bladder actually contains two sets of cell membranes in series, these membranes have the same relative permeability coefficients and may therefore be regarded as forming a single membrane for the purpose of computing tracer fluxes. The expression for the one-way flux M_x of the ion x at electrochemical equilibrium is

$$M_x = RTG_x/z_x^2F^2 = \frac{RTP_xC_x}{F^2\Omega\sum_n P_nC_nz_n^2},$$

where G_x is the partial conductance of x, Ω the membrane resistance, and P_x the relative permeability coefficient (e.g. Hodgkin, 1951). Substituting experimental values for Ω and P derived from the constant-field equation, one obtains the following predictions for passive one-way fluxes in NaCl

or NaBr solutions: Na 7·8, K 0·24, Br 0·55 μmole/cm^2.hr. The experimental values were: Na 8·4 $\pm$ 2·4, K 0·30–0·36, Br 12·2 $\pm$ 2·5 μmole/cm^2.hr.

For K and Na the agreement between tracer fluxes and predictions based on resistance and diffusion potentials is good, and the passive fluxes of these ions may consequently be attributed to simple diffusion. In line with this conclusion are the facts that neither the passive fluxes nor the diffusion potentials were directly affected by cyanide-iodoacetate; and that replacement of all luminal Na by K had no effect on the Na influx. Such a K–Na substitution might have decreased the Na influx if there were an exchange-diffusion component, and increased it if single-file effects were taking place. It is still uncertain, however, whether the increase of luminal K in some experiments is due to transport into the lumen or to leakage from the tissue. A similar increase of luminal K in guinea-pig gall-bladder *in vitro* appears to result from both causes combined (Herman, Wilson & Kazyak, 1958). The effect is in any case minute, since it is not observed in 50 % of all experiments, and in the remaining 50 % the net K 'influx' averages less than one fiftieth of the net Na efflux.

For Br the measured fluxes and the predictions of simple diffusion are in complete disagreement, and most of the passive Br flux cannot be electrically conducting or due to simple diffusion. The existence of a non-conducting exchange-diffusion flux was also indicated by the low flux ratio when luminal NaBr was partially replaced by sucrose; and by the sensitivity of the passive flux to cyanide-iodoacetate, which has no effect on permeabilities calculated from diffusion potentials, hence on simple diffusion of Br. A further indication that the electrical conductance due to halide anions is slight, compared with that due to Na, is that sulphate–chloride replacements had no detectable effect on the resistance.

Since one-for-one exchange diffusion cannot give rise to a net flux, it is still possible to compare Br permeability from diffusion potentials and resistance measurements with Br tracer fluxes by restricting consideration to a net Br flux, which must be electrically conducting. The form of the constant-field equation that relates net fluxes M to the absolute permeability $\bar{P}$ ($\bar{P} = GRT/CF^2$) is

$$M = \frac{\bar{P}FV\{C^o - C^i \exp(-F\Delta\psi/RT)\}}{RT\{1 - \exp(-F\Delta\psi/RT)\}}.$$

For the situation where the poisoned gall-bladder separates NaBr from 50 % NaBr + 50 % sucrose, this predicts a net flux of 2·0 μmole/cm^2.hr, in good agreement with the observed value of 2·2 μmole/cm^2.hr.

Thus, except for the additional contribution to the Br flux from exchange diffusion, the constant-field equation predicts Na, K and Br tracer fluxes rather well. The flux results are summarized in Fig. 7, along

with water fluxes obtained in the following paper. Since the pump efficiencies, permeability coefficients, and intracellular concentrations of Br and Cl are about the same, it would be surprising if exchange diffusion did not apply to Cl as well as to Br.

There remains the problem of interpreting the relative permeabilities. To summarize the experimental evidence, the permeabilities to K^+ and Na^+ are high, to Cl^-, Br^-, $CH_3SO_4^-$, TEA^+, and sucrose low, and to SO_4^{2-} and bilirubin zero. If one takes the hydrated diameter of K as 3 Å (Conway, 1957) and uses the relative hydrated diameters of various ions calculated from limiting ionic conductances by Coombs, Eccles & Fatt (1955) and Araki, Ito & Oscarsson (1961), the hydrated diameters of K, Na, Cl, Br, CH_3SO_4, TEA and SO_4 are 3·0, 4·4, 2·9, 2·8, 6·9, 7·8 and 5·6 Å,

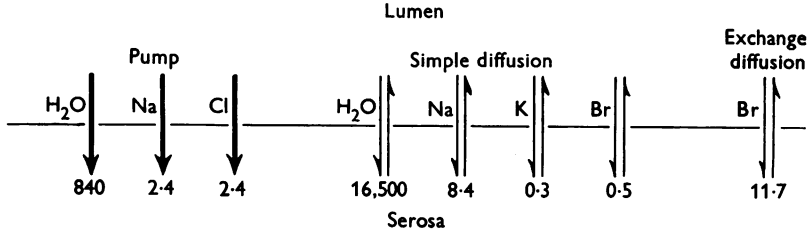

Fig. 7. One-way fluxes of substances across the gall-bladder (μmole/cm^2.hr) when both sides are bathed in NaCl (or NaBr) Ringer's solution, as measured by radioactive tracers. Pump refers to net fluxes caused by the active-transport mechanism. Simple diffusion and exchange diffusion produce equal and opposite fluxes in both directions.

respectively. These estimates are of course approximate but are of interest chiefly for comparative purposes. Since Cl and Br are if anything slightly smaller than K and Na but have much lower permeabilities, it is probable that the gall-bladder contains negative fixed charges, whose existence will in fact be demonstrated more directly in the next paper (Diamond, 1962*b*). These charges would account for low permeability to monovalent anions regardless of size, and zero permeability to the divalent anion sulphate. This explanation cannot hold for the cation TEA and the uncharged bilirubin, and, as these are the largest molecules tested, there must also be a steric factor reducing and eventually abolishing permeability to molecules of diameter over, say, 7 Å. This factor would also explain why Ca^{2+} (7·5 Å), though positively charged, must nevertheless have a very low permeability, to judge from comparative analyses of hepatic and gall-bladder bile. The physiological advantages of the steric factor in preventing bilirubin and bile-salt losses from gall-bladder bile are obvious.

Mode of transport of Na and Cl

The question of whether Na and Cl are transported actively may now be considered quantitatively. A sufficient condition for assuming active transport of an ion is that net movements of the ion occur against its electrochemical gradient $\Delta\mu$, which is given by the following expression in an isothermal system at constant pressure:

$$\Delta\mu = RT \ln (a_l/a_o) + zF\Delta\psi. \quad (4)$$

Equation (4) assumes that solvent-drag effects are quantitatively negligible, and this omission will be justified in the next paper.

It has been shown that net absorption ceases only when 93 % of the luminal NaCl has been replaced by sucrose. In the situation where, for example, 80 % of luminal NaCl has been replaced by sucrose, substitution of experimental values for $\Delta\psi$ and a in eqn. 4 yields $\Delta\mu_{Na} = -650$ volt-coulombs mole, $\Delta\mu_{Cl} = -6320$ volt-coulombs mole. The minus sign means that the lumen is at a lower electrochemical potential than the outer solution for both Na and Cl, and both Na and Cl would accordingly move inwards if subject only to passive forces. Since the net movements of both are in fact outwards, both Na and Cl must be actively transported. The same conclusion is reached if the calculation is made for any luminal replacement up to 93 %.

One may express the meaning of this calculation in another way. When luminal NaCl is partially replaced by sucrose, the lumen goes electrically positive because of the diffusion potential determined largely by Na. Thus, the net outwards movement of Cl is proceeding against both a concentration gradient and an electrical potential, and Cl transport must be active. Na is moving against a concentration gradient, but with the p.d. However, because the gall-bladder has a finite permeability to K and Cl, the p.d. is always less than that predicted for an Na electrode ($RT \ln (a_o/a_l)$), and therefore insufficient to balance the concentration gradient. Thus, the transport of Na from NaCl–sucrose mixtures is active.

Another test of the nature of Na transport is provided by the case of 50 % KCl + 50 % NaCl in the lumen and NaCl as the outer solution. Under these circumstances the p.d. is virtually nil ($-0{\cdot}8 \pm 0{\cdot}7$ mV), but a_{Na} is twice as high in the outer solution as in the lumen. Since a net efflux of Na was still observed, Na transport in this case is also against the chemical potential and by definition active.

Relation between Na transport and Cl transport

In all epithelial membranes which have been investigated in detail to date, active transport of ions has been shown to be associated with an

externally measurable p.d. While the pump mechanisms themselves need not be directly electrogenic, these p.d.s do demonstrate that the pumps are independent, in the sense that active transport of the ion involved does not directly require transport of its counter-ion. Even in the three preparations (stomach, small intestine, *in vivo* frog skin) which, like the gall-bladder, perform an active transport of two oppositely charged ions in the same direction, each pump produces a p.d. independently. In the gall-bladder, however, the failure to observe a p.d. in Na_2SO_4, $NaCH_3SO_4$, TEACl, and KCl seemingly implies that active transport of Na cannot occur without active transport of Cl. Before this conclusion can be accepted, it is essential that the argument against independent pumps should first be made quantitative: i.e. one must calculate whether independent pumps would produce a p.d. large enough to detect, if they existed.

The calculation requires knowledge of the ionic conductances (G) and E^*, the electromotive force associated with active transport of an ion (Ussing & Zerahn, 1951). E^* is the p.d. that one would actually measure if no other ion were actively transported and if the membrane were impermeable to all other ions. Two methods are available for computing E^*. In the first place, if the passive flux of an ion is due only to simple diffusion, E^* may be calculated from the flux ratio at electrochemical equilibrium ($RT \ln a_i/a_o + zF\Delta\psi = 0$) by the equation (Ussing & Zerahn, 1951) $E^* = RT/F \ln (M_i/M_o)$. With NaCl Ringer's solution on both sides of the preparation, electrochemical equilibrium obtains, and M_o and M_i are 10·8 and 8·4 μmole/cm^2.hr, respectively, for Na. Thus, $E^*_{Na} = 58 \log 8{\cdot}4/10{\cdot}8 = -6{\cdot}3$ mV. This method may not be applied to Br, since most of the Br passive flux represents exchange diffusion.

The second method of calculating E^* arises from the definition of the partial conductance of an ion, $G_x = \partial I_x/\partial\psi$, where I_x is the current density of the ion x. When the ion x is at electrochemical equilibrium, the residual net current is due to active transport and given by $G_x E^*_x = I_x = M^*_x z_x F$, where M^*_x is the net active flux. Thus

$$E^*_x = M^*_x z_x F/G_x \tag{5}$$

which is analogous to Ohm's Law. M^* must be measured under conditions of electrochemical equilibrium. This condition is satisfied in the weighing experiments, where the luminal and outer solutions were identical and the p.d. averaged under 1 mV. Thus M^* was calculated from the rates of weight loss in the various solutions given previously (Diamond, 1962*a*), assuming (as was proved for NaCl and Na_2SO_4) that the absorbate is isotonic. Gs were calculated from the relative permeability coefficients and resistance. Es were then computed from eqn. (5); for instance, $G_{Cl} = 0{\cdot}34$ mmho/cm^2 and $M^*_{Cl} = -2{\cdot}4$ μmole/cm^2.hr in NaCl Ringer's solution; $z_{Cl} = -1$, $F = 96{,}500$ C; thus,

$$E^*_{Cl} = \frac{(-1) \times (96{,}500) \times (-2{\cdot}4 \times 10^{-6})}{(0{\cdot}34 \times 10^{-3}) \times (3600)} = +189 \text{ mV}.$$

The sign indicates that transport of Cl from the lumen leaves the lumen positive. Similarly, the other values are: $E^*_{Na} = -7{\cdot}8$ mV, $E^*_{Br} = +110{\cdot}2$ mV, $E^*_{CH_3SO_4} = +44{\cdot}9$ mV, and $E^*_{TEA} = -25{\cdot}3$ mV. Since P_{SO_4} is experimentally indistinguishable from zero, G_{SO_4} is nominally zero and $E^*_{SO_4}$ infinite, and one must write $E^*_{SO_4} = z_{SO_4} F M^*_{SO_4}/G_{SO_4}$, where G_{SO_4} is an arbitrarily small quantity. $E^*_{Na} = -6{\cdot}3$ mV, as calculated by the first method, is in good agreement with $E^*_{Na} = -7{\cdot}8$ mV from the second method.

Figure 8 shows the equivalent electrical circuit when two ionic species are actively transported. E_1 and R_1 are E^* and $1/G$ for one actively transported ion, and E_2 and R_2 are the

same quantities for the other. R_3 is the passive leak due to all passive ions, of which all but potassium may be ignored in the gall-bladder. The open-circuit p.d. for this network is

$$\frac{(E_1^* R_2 + E_2^* R_1)\, R_3}{R_1 R_2 + R_2 R_3 + R_3 R_1}. \tag{6}$$

This approach may be illustrated by calculation of the expected p.d. in TEACl solution. If TEA is taken as species 1 and Cl as species 2, then it is known that

$$E_1^* = -25{\cdot}3 \text{ mV}, \quad R_1 = 1/G_{\text{TEA}} = 6250\ \Omega\ \text{cm}^2, \quad E_{\text{Cl}}^* = +189 \text{ mV},$$

$$R_2 = 1/G_{\text{Cl}} = 2940\ \Omega\ \text{cm}^2, \quad R_3 = 1/G_{\text{K}} = 3840\ \Omega\ \text{cm}^2.$$

When substituted in eqn. 6, these values yield +79·3 mV—i.e. the lumen should be 79·3 mV positive in TEACl solution. This may be regarded as the p.d. of the chloride pump shunted by parallel TEA and K resistances, and reduced another 6 % by the weak TEA pump of opposite sign. Equation (6) also predicts 0·0 mV for NaBr solution, 0·0 mV for NaCl, −5·5 mV for $NaCH_3SO_4$, −5·5 mV for Na_2SO_4, and +7·5 mV with KCl in the lumen and NaCl outside.

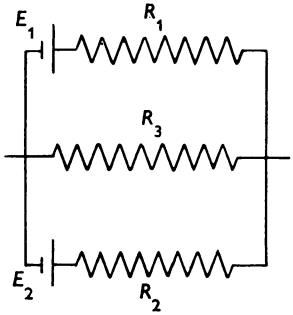

Fig. 8. Electric circuit. For explanation see text.

Thus, if the Cl pump is independent, active transport of Cl outwards should leave the lumen positive by 79·3 mV in TEACl and by 7·5 mV with KCl in the lumen, whereas the experimental values are $-0{\cdot}3 \pm 0{\cdot}3$ mV and $+0{\cdot}3$ mV, respectively. Similarly, if the Na pump is independent, the lumen should be 5·5 mV negative in $NaCH_3SO_4$ and Na_2SO_4, but the experimental values are −1·5 mV and $-1{\cdot}7 \pm 0{\cdot}9$ mV. The p.d. in Na_2SO_4, though much too low quantitatively, is at least in the right direction qualitatively for an outwards-directed Na pump. However, this p.d. is not affected by cyanide-iodoacetate, and whatever its origin, is not likely to be related to pumping. The p.d. of −0·3 mV in TEACl is not even of the correct sign for a Cl pump. In any case, $-1{\cdot}7 \pm 0{\cdot}9$ or $-0{\cdot}3 \pm 0{\cdot}3$ mV are too close to zero experimentally to offer a secure basis for any theories.

Thus, there is a complete discrepancy between the large p.d.s expected for independent pumps and the negligible p.d.s actually observed. This contrasts with the situation in frog skin, where p.d.s of 120–150 mV expected for an independent Na pump are actually observed in sulphate solution (Koefoed-Johnsen & Ussing, 1958). The simplest explanation for this discrepancy in the gall-bladder is that the Na and Cl pumps are not

independent and can function only in each other's presence. However, some more orthodox hypotheses must also be examined: (1) Ions other than Na and Cl may exert non-specific damaging effects. This hypothesis is refuted by the fact that high diffusion potentials, a sensitive criterion of the state of the preparation, persist for up to several hours in other solutions. Furthermore, gall-bladders that have effected little fluid absorption in Na_2SO_4 or $NaCH_3SO_4$ absorb at a normal rate when transferred to NaCl. (2) The substitution ions may never penetrate the cells and never be available for pumping. However, K, which is not absorbed, is a normal intracellular constituent, while one can calculate that the half-time for exchange of CH_3SO_4 and TEA with the intracellular fluid is around 1 min. (3) Since solutions of substitution ions have usually been present on both sides of the gall-bladder, luminal absorption might require the presence of NaCl in the outer solution. However, NaCl is absorbed from the lumen with Na_2SO_4 in the outer solution, and there is no improvement in Na_2SO_4 absorption from the lumen with NaCl in the outer solution. (4) Na absorption might require only the presence of Cl in the lumen, and vice versa. This assumption could be maintained only if an agent could be found which inhibits one pump and not the other, thus producing partial inhibition of absorption and appearance of a p.d. due to the surviving pump. However, ouabain, oxytocin, and cyanide-iodoacetate inhibited absorption completely, and the partial inhibitor cyanide had no effect on the p.d. (5) There might be active transport of water, carrying salt across by solvent drag. However, it will be shown in the next paper that even if there were active transport of water, the solvent-drag effect would be too low by a factor of 14; that a solvent-drag mechanism would set up a p.d. because P_{Cl} is much less than P_{Na}; and that in any case there is apparently no active transport of water.

Even if one ignores the evidence from other salts, the absence of a p.d. in NaCl Ringer's solution demands that independent Na and Cl pumps would have to operate fortuitously at the same rates. Since the p.d. at lower values of luminal [NaCl] still behaves as a simple diffusion potential, the assumption of independent pumps would demand that both pumps fortuitously also had the same saturation kinetics, and that the same coincidences existed in bromide solutions. The likelihood of these coincidences seems remote, and they would still not account for the absence of a p.d. in solutions based on four other salts. The simplest explanation of the failure to measure an active transport potential under any circumstances is that there exists an obligate direct coupling between transport of Na and Cl, rather than the usual electrical coupling. The pump must transfer NaCl across the cell membrane as a neutral molecule—i.e. the transport of one sodium ion and one chloride ion must be directly linked.

Mechanism of salt pumping

The following argument shows that the pump must at least in part be located at the outer border of the epithelial cells. Since semi-permeability and virtually the whole resistance to movement of ions have been shown to be properties of the epithelial cells but not of the serosa, the salt pump must be associated with the epithelial cells, as assumed in other epithelial membranes. The situation in NaCl Ringer's solution may be represented as follows:

Outer solution		Cell		Luminal solution	
Na	144 mM	Na	54 mM	Na	144 mM
K	4·5	K	81	K	4·5
Cl	149·6	Cl	91	Cl	149·6

From the known permeability coefficients the resting potential of the cells must be practically nil. Since both Na and Cl are moving down concentration gradients from lumen to cell and against concentration gradients from cell to outside, both Na and Cl must be actively transported at the outer membrane. It is encouraging that the same conclusion was also reached from totally different evidence, namely, the ability of the cells to pump out part of their volume during absorption transients after removal of luminal NaCl (Diamond, 1962*a*).

In addition to producing no transport p.d., the gall-bladder differs from some other epithelial preparations as to the symmetry of its diffusion potentials. Asymmetrical permeability plays an essential role in the most thoroughly investigated epithelium, frog skin, which performs an active transport of NaCl from the outside to the inside (Koefoed-Johnsen & Ussing, 1958). The outer membrane of an epithelial cell is relatively permeable to Na but impermeable to K, while the inner membrane is relatively permeable to K but impermeable to Na. A coupled K–Na pump on the inner membrane transfers K into the cell and Na out of the cell. Thus, the active transport potential, which carries Cl passively inwards, is the sum of a K diffusion potential at the inner membrane and an Na diffusion potential at the outer membrane. Kidney proximal tubule seems to operate by a similar pump and membrane asymmetry (Giebisch, 1961). However, in gall-bladder P_K equals P_{Na} at both the luminal and serosal membranes. If, as postulated in frog skin, one of the membranes contained a coupled K–Na pump, it could not cause an active-transport p.d. or net transfer of cation, and would only exchange the Na of the lumen against the K of the outer solution. Whatever the mechanism of salt transport, the observed unilaterality of the K-free effect (only on the outside of the gall-bladder) cannot mean that Na transport is directly attributable to a coupled K–Na pump in the outer membrane. Possibly a K–Na pump in

the outer membrane of the gall-bladder simply maintains high intracellular [K], which may be indirectly necessary for salt transport. The K-free effect on anion transport in numerous tissues must similarly have an indirect explanation.

One can envisage a wide variety of mechanisms which would effect a neutral transport of NaCl and bear no resemblance to standard epithelial postulates. However, the gall-bladder is distinguished not only by its unique absence of an external p.d. but also by some striking similarities to other epithelia—the inhibitory ouabain effect, K requirement, specificity for NaCl, saturation kinetics, and isotonic water transfer. These experimental parallels with other preparations initially limit the acceptable mechanisms to those involving the minimum number of new assumptions necessary to explain the apparent neutrality.

The simplest hypothesis is that a Na transport site and Cl transport site are located on the same carrier molecule in the outer membrane, and that this double carrier can cross the membrane only when both sites are occupied. Such a double carrier would directly effect a neutral transport of NaCl—i.e. one Na ion to every Cl ion—and neither binding site could operate in the absence of the counter-ion. Thus, a p.d. could not be produced under any circumstances, even in solutions based on salts such as TEACl, Na_2SO_4, or $NaCH_3SO_4$, where either Na or Cl is absent. The appearance of features common to other epithelia, such as saturation kinetics and specificity for Na and Cl, would still be due to the presence of normal Na and Cl transport sites.

An alternative hypothesis derives from the observation, made in a preliminary study with the electron microscope, that gall-bladder epithelial cells appear to be full of vesicles. Hence one might suppose that normal Na and/or Cl transport sites are located on the surfaces of these vesicles and serve to fill them with NaCl isotonic to the intracellular fluid. The vesicles then move to the outer membrane and discharge their contents to the outside. Conventional active-transport p.d.s might be set up between the inside of the vesicles and the rest of the intracellular fluid, but there would be no p.d. between opposite ends of the cell. However, until more is known about the properties of the observed vesicles, such a hypothesis will involve numerous *ad hoc* assumptions, whereas the only new postulate of the double-carrier hypothesis is that Na and Cl binding sites are directly coupled.

Finally, it may be helpful to review the barriers to active and passive ion movements in the gall-bladder. An ion which completely traverses the gall-bladder passes through four structures: a negatively charged cell membrane at the luminal border of the epithelial cells, the intracellular contents of the epithelial cells, another negatively charged cell membrane

at the outer border of the epithelial cells, and the serosa, which retards the diffusion of ions indiscriminately. The passive properties of the gall-bladder in NaCl Ringer's solution are such that most of the resistance lies in the two sets of cell membranes, and most of the conductance is due to sodium. The passive movements of ions may be attributed to simple diffusion, except that bromide may also cross the cell membranes by exchange diffusion; and the outer cell membrane contains a neutral mechanism for active transport of NaCl.

SUMMARY

1. The mechanism of NaCl transport in the *in vitro* gall-bladder of fresh-water fish has been studied by electrical methods and use of radioactive tracers.

2. When both sides of the gall-bladder are bathed by identical solutions based on NaCl, NaBr, Na_2SO_4, $NaCH_3SO_4$, or tetraethyl ammonium chloride (TEACl), the electrical potential difference across the organ is practically nil. This contrasts with the active-transport potentials observed in all other epithelia analysed so far.

3. From diffusion potentials between non-identical solutions, the relative permeability coefficients are: $P_{Na}:P_K:P_{Br}:P_{CH_3SO_4}:P_{Cl}:P_{TEA}$: $P_{CH_3SO_4} = 1{\cdot}00:1{\cdot}00:0{\cdot}07:0{\cdot}05:0{\cdot}04:0{\cdot}02:0{\cdot}00$. These suggest the presence of negative fixed charges.

4. The transients observed in diffusion potentials when a bathing solution is suddenly changed imply that the serosa retards the diffusion of all ions indiscriminately.

5. The resistance is $113 \pm 16\ \Omega\ cm^2$.

6. The one-way passive fluxes of Na, K, and Br agree well with predictions of the constant-field equation and simple diffusion, except for the additional operation of an exchange-diffusion mechanism for Br.

7. Transients in the radioactive fluxes imply that ions crossing the gall-bladder actively or passively go through the epithelial cells.

8. Both Na and Cl are actively transported. However, if the transport mechanisms were independent, voltages of 5–79 mV would have been observed.

9. Since no such voltages are observed, the gall-bladder provides the first instance of a neutral NaCl pump, in which transport of one Na ion is directly coupled to transport of one Cl ion. The simplest explanation is that Na and Cl transport sites occur on the same carrier molecules.

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